DRAFT

Working Group 1d. Bile, Bilirubin and Cholestasis

Introduction & Background

Among the many functions of the liver, production of bile is the most distinctive and liver-specific. Adult humans produce ~500 ml of bile per day, which is an aqueous solution containing bile salts, cholesterol, phospholipids, proteins, bilirubin conjugates, and other solutes. Bile facilitates digestion and absorption of lipids and cholesterol, and also functions as the major vehicle for elimination of cholesterol from the body. Following its synthesis by hepatocytes, bile is secreted into and modified in bile ducts before entering the gallbladder, where it is concentrated and stored. After meals, the gallbladder contracts, and bile flows through the cystic and common bile ducts into the intestine, where it mixes with food and helps to solubilize and absorb fats. Bile salts are then actively reabsorbed in the distal small bowel and taken up by the liver from the portal blood as a part of the enterohepatic circulation. Cholestasis reflects the retention of bile caused by mechanical or biochemical restriction of bile flow. Liver conditions with prominent cholestasis are called cholestatic liver diseases. Basic research on bile formation and flow provides not only important understanding of this liver function, but also insights into the mechanisms of cholestasis and the necessary basis for developing therapies to ameliorate or prevent cholestatic liver diseases.

Production of bile is a complex process comprising: 1) hepatic uptake of bile salts, bilirubin, cholesterol and other solutes (e.g., porphyrins, drugs, toxins, endorphins); 2) conjugation or metabolic modification of selected solutes; 3) transport or diffusion of the compounds across the hepatocyte to the bile canaliculus; 4) simultaneous regulated *de novo* synthesis of bile salts, phospholipids and cholesterol; 5) secretion of bile salts, cholesterol, phospholipids and conjugated organic solutes across the canalicular membrane; 6) formation of bile in the bile ducts; and 7) flow of bile to the gallbladder and duodenum. Disruption of one or more of these processes can cause cholestasis or jaundice and result in retention of bile, liver cell injury and progressive liver fibrosis. For example, progressive familial intrahepatic cholestasis type 2 (PFIC-2) is an inherited disease with onset in infancy caused by mutations in the bile salt export pump (BSEP), which results in interrupted secretion with consequent intracellular retention of bile salts (#5 above). Primary biliary cirrhosis is a cholestatic disease of adults characterized by gradual inflammatory destruction of small intralobular bile ducts, which results in interruption of bile flow from the canaliculus to the major bile ducts (#7 above). Other important cholestatic liver diseases include biliary atresia, PFIC-1 & -3, Alagille syndrome, intrahepatic cholestasis of pregnancy, many drug-induced liver diseases, graft-vs-host disease, idiopathic vanishing bile duct syndrome, cholestasis of sepsis and total parenteral nutrition, primary sclerosing cholangitis, and biliary obstruction from gallstones, stricture and cancer. Genetic diseases of bilirubin metabolism, including Gilbert syndrome, Crigler-Najjar syndrome (types 1 and 2) and Dubin-Johnson syndrome, also result in jaundice, but without cholestasis and liver cell injury.

Recent Research Advances

Research on bile, bilirubin and cholestasis is important in helping to define the primary causes of cholestatic liver disease, as well as in identifying means to prevent the complications of bilirubin and bile retention. Aided by the Human Genome Project and application of the powerful tools of molecular biology, important advances have been made in elucidating each of the processes of bile production and secretion.

Uptake of bile salts, bilirubin, cholesterol and organic solutes by the hepatocyte: Several of the key transporters for hepatic uptake of bile salts, bilirubin, lipids and organic solutes in humans have been cloned and characterized biochemically including those for conjugated bile salts (NTCP), at least four organic anion transporters (OATPs), and an organic cation transporter (OCT1). Expression of these uptake proteins and other hepatic transporters is regulated largely by recently identified members of the orphan nuclear receptor superfamily that are ligand activated and form heterodimers with the multi-function nuclear receptor RXR, including RAR (for vitamin A), FXR (for bile acids), CAR (for bilirubin), LXR (for sterols), and PXR (for xenobiotics such as St. John's Wort). Other members of this superfamily are also involved in control of hepatobiliary transporter gene expression, but bind to DNA response elements as monomers (e.g., SHP, LRH1, HNF4α). Studies of these transporters and their regulatory pathways will provide insights into the pathophysiology of several cholestatic liver diseases and new approaches to treatment. Indeed, molecular analyses of CAR and its actions on bilirubin metabolism have suggested that the ancient Chinese herbal medication Yin Zhi Huang, used to treat neonatal jaundice, has a physiological basis in that it induces bilirubin uptake, conjugation and secretion into bile through activation of CAR.

Conjugation or metabolic modification of organic solutes and transport across the hepatocyte to the site of metabolism or secretion: Fat-soluble and amphiphilic compounds taken up by the liver are ultimately converted to more water-soluble forms by enzymatic oxidation and conjugation reactions. This enhanced solubility in water facilitates their secretion into bile after transport across the canalicular membrane or into the urine after secretion into the blood. Bilirubin, for example, is conjugated with glucuronic acid then secreted as a mono- or diglucuronide in bile. The enzymatic pathway for bilirubin conjugation has recently been defined, and mutations in the genes coding for these enzymes can have severe consequences because the unconjugated form of bilirubin is neurotoxic. For example, the severe neonatal disease Crigler-Najjar syndrome is caused by inherited defects in a specific UDP-glucuronosyl transferase (UGT1A1). Lack of this enzymatic activity to conjugate bilirubin leads to an inability to clear it and marked indirect hyperbilirubinemia presenting in the newborn period. A transient lack of bilirubin conjugation due to low initial UGT activity is also responsible for the mild hyperbilirubinemia and jaundice of newborns. Mutations in the proximal promoter of the UGT1A1 gene (additional thymines and adenines in the TATAA box) cause a more modest reduction in the enzyme's activity and result in the common condition of mild hyperbilirubinemia, known as Gilbert syndrome.

Synthesis of bile salts and cholesterol: Much progress has been made in characterizing the enzymatic pathways of bile acid and cholesterol synthesis and how these pathways are regulated. The classic bile acid biosynthetic pathway leads to synthesis of cholic and chenodeoxycholic acid from cholesterol. An alternate or acidic pathway of bile salt synthesis contributes little normally, but becomes important during liver injury. Regulation of bile salt synthesis is also dependent upon nuclear receptors (e.g., RXRα, HNF4α, LRH1, LXR, FXR, SHP, PXR, and VDR) and is coordinated with hepatic uptake and canalicular secretion of bile salts, cholesterol and phospholipids. Mutations in several of the enzymes that participate in bile salt synthesis have been identified as causes of human disease. For example, mutations in the CYP27A1 gene, the first step in the alternative pathway for bile salt synthesis, result in cerebrotendinous xanthomatosis (CTX). Particularly exciting are recent discoveries that bile salts may regulate lipid metabolism and gluconeogenesis in response to fasting, via nuclear receptors and their coregulators (e.g., PGC1α). Thus, pathways of bile salt production intersect with regulatory pathways for glucose and cholesterol metabolism, and are important in obesity, gallstone formation, diabetes and atherosclerosis.

Canalicular secretion of cholesterol, bile salts, bilirubin and organic solutes: Bile salts, cholesterol, phospholipids, bilirubin and other organic solutes are transported out of the hepatocyte into the canaliculus by specific transport proteins. Over the past several years, many of these transport proteins have been identified and characterized, including MDR1/ABCB1 (for organic cations), MRP2/ABCC2 (for drug conjugates), ABCG5 & 8 (for cholesterol), MDR3/ABCB4 (for phosphatidylcholine), BCRP/ABCG2 (for drugs & porphyrins), and BSEP/ABCB11 (for bile salts). Inherited defects in several of these transporters have been linked to various diseases, including progressive familial intrahepatic cholestasis (PFIC). FIC1 is an amino-phospholipid transferase that translocates phosphatidylethanolamine and phosphatidylserine from the outer to the inner bilayer of plasma membranes. Defects in the *FIC1* gene can result in Byler disease (PFIC-1), while other mutations in the same gene cause benign recurrent intrahepatic cholestasis (BRIC). Defects in the *BSEP* gene are linked to the second form of Byler disease (PFIC-2) while mutations in the *MDR3* gene underlie PFIC-3. Mutations and polymorphisms in *MDR3* have also been described in adults with cholestasis of pregnancy, intrahepatic gallstones and idiopathic progressive cholestasis. Characterization of these transporters and their function has facilitated diagnosis of the PFIC syndromes and could ultimately provide approaches to their therapy or prevention.

Formation of bile and bile flow: Bile is formed in the biliary canaliculus as a micellar aqueous solution containing predominantly bile salts, cholesterol, and phospholipids with lesser amounts of bilirubin glucuronides, calcium and bile proteins. Characterization of the physical chemical composition of bile has helped in the understanding of both cholesterol and bilirubinate gallstone formation and the complications of cholestasis. Bile flow is maintained by active secretion of organic solutes and the secretion of fluid and electrolytes. Recently, biliary cells have been found to possess cilia and motor complexes that act as sensory organelles and participate in regulating electrolyte secretion and bile flow. Defects in biliary cilia appear to underlie inherited syndromes of

polycystic liver disease. Abnormalities of electrolyte transport, such as those present in cystic fibrosis, can cause bile stasis and lead to chronic liver disease and cirrhosis.

These dramatic discoveries in defining the mechanisms of bile production and secretion provide important insights into cholestatic liver diseases. Further research promises to provide new approaches to treatment and prevention of these diseases.

Research Goals

The ultimate goals for research on bile, bilirubin, and cholestasis are to fully delineate the normal pathways of uptake, metabolism and secretion of bile salts, bilirubin, and other biliary lipids and solutes; to characterize the alterations in these pathways that participate in the pathogenesis of liver diseases; and to develop means of diagnosis, treatment, and prevention of cholestatic liver disease and disorders of bilirubin metabolism.

Basic Research: Continued basic laboratory research directed toward the goal of more fully defining the normal physiology and regulation of bile formation, and cholesterol synthesis and catabolism would be helpful (Matrix Cell B2). This is a broad and challenging goal for future research, but it is critical for further advances in diagnosis, management and prevention of cholestatic liver diseases. Thus, it is important to fully identify the sinusoidal transporters responsible for the uptake of bile salts, cholesterol, lipids, and other organic solutes, as well as intracellular proteins in the cytosol and endoplasmic reticulum responsible for their transcytoplasmic transport and canalicular secretion. Furthermore, the mechanisms of action and regulation of these various types of transporters require assessment. These mechanisms include transcriptional and post-transcriptional regulation of the transporters as well as interactions with cell signaling pathways in hepatocytes. The roles of aquaporins and purinergic regulation in the formation of bile also deserve further study. Finally, the pathways and regulation of bile acid, cholesterol and lipid synthesis in hepatocytes require complete delineation, including how all these processes are coordinated in regulation of bile formation and secretion.

Because bilirubin is normally secreted in bile, cholestatic liver diseases are typically associated with bilirubin retention and jaundice. However, bilirubin elevations can occur without cholestasis due to overproduction of bilirubin and/or abnormalities in bilirubin uptake, conjugation and secretion. Abnormalities in bilirubin metabolism independent of cholestasis occur in the benign Gilbert and Dubin-Johnson syndromes, as well as the severe and life-threatening Crigler-Najjar syndrome. Hyperbilirubinemia without cholestasis can also occur in the neonatal period associated with immaturity of liver function and the bilirubin metabolic pathways, often complicated by the added stresses of prematurity, sepsis, electrolyte imbalance and malnutrition. Some degree of hyperbilirubinemia in the neonatal period is “physiologic,” but severe hyperbilirubinemia can lead to kernicterus, permanent neurologic damage, and death. Better understanding of the maturation of hepatic functions during fetal life would enable improved understanding of the pathophysiology that leads to severe neonatal jaundice and

kernicterus (Matrix Cell A3). Ideally, with the development of reliable and noninvasive means of assessing the maturity of the bilirubin metabolic pathways safe and effective means of preventing or ameliorating neonatal hyperbilirubinemia would be possible (Matrix Cell B3).

Pathophysiology of Cholestatic Liver Disease: Knowledge of the genes associated with inherited diseases does not always translate readily into advances in diagnosis or therapy. To date, this translation gap has existed for the genes associated with neonatal cholestatic syndromes. An important goal for future research is the elucidation of the structure-function relationships of the various transporters and enzymes found to be abnormal in cholestatic liver diseases and the development of potential targets for small molecule therapy (Matrix Cell A2). Representative issues include: How does *Jagged-1,* which is mutated in Alagille syndrome, cause progressive bile duct loss, and how might this might be reversed or ameliorated by modulation of other pathways involved in Notch signaling? How do the mutations in the *FIC1* gene lead to bile secretory failure, and how might this be prevented or reversed? Elucidation of the pathophysiology behind cholestatic liver diseases could be followed by studies aimed at translation of these findings to practical clinical use. In addition, the genes that cause some forms of severe neonatal cholestatic liver disease have not yet been identified, and, therefore, the elucidation of these genes, including their gene products and their regulation, is a major goal for future research (Matrix Cell A1). Another important goal with therapeutic implications is the elucidation of adaptive response mechanisms of efflux transporters for bile salts and xenobiotics in hepatocytes and cholangiocytes during cholestasis. A centralized registry of colonies of knock-out and mutant animal models related to cholestatic liver disease would also be useful.

While many genes involved in bile acid uptake, transport, synthesis and secretion are found to be abnormal in neonatal inherited cholestatic syndromes, the roles of these genes in adult or acquired forms of cholestatic liver disease are not well defined. Thus, idiopathic cholestasis of pregnancy is a syndrome of unknown cause that results in cholestasis, pruritus and liver disease during the last two trimesters of pregnancy and can result in fetal loss or prematurity. This syndrome has a strong familial component and is particularly common in certain ethnic groups, including individuals of Scandinavian and Chilean descent. Some cases of idiopathic cholestasis of pregnancy are associated with heterozygosity for mutant forms of *MDR3* and *BSEP*. Further elucidation of the genetic components of this syndrome, as well as other cholestatic syndromes (e.g., vanishing bile duct disease, cholestatic drug-induced liver disease), is important and could lead to the development of means for early and accurate diagnosis, prevention and treatment (Matrix Cells B1 and C2). Full definition of the pathways of bile uptake, synthesis and secretion may also allow for understanding of the cholestasis of sepsis, shock and total parenteral nutrition and lead to identification of means of prevention or treatment of these syndromes.

Clinical Investigation: Identification of the causes of cholestatic liver disease may facilitate the future development of accurate, noninvasive ways to diagnose these conditions, including application of rapid mass spectrometric methods. In addition, if

cholestatic reactions to medications, estrogen therapy or pregnancy are associated with polymorphisms of bile salt and organic anion transporters, there is a potential that screening tests for these idiosyncratic reactions could be developed (Matrix Cell C2). Identification of these molecular mechanisms underlying acquired cholestasis can also aid in elucidating the mechanisms of action of hepatoprotective drugs and in correctly applying these drugs to specific diseases.

An important clinical symptom associated with cholestatic liver disease is itching or pruritus. Pruritus is typical of chronic cholestatic liver disease and can be severe and incapacitating. Indeed, intractable pruritus is a reason for liver transplantation in a proportion of patients with cirrhosis. The underlying cause of pruritus is unknown. While initially thought to be due to bile retention and excessive levels of bile salts in nerve endings in the skin, this hypothesis remains unproven. An important research goal is to identify the molecular mechanisms that cause the sensation of itch and to develop specific targets that might be used for therapy of pruritus (Matrix Cell C1).

Therapy: Ultimately, safe and effective drug therapies for cholestatic liver diseases would be of great benefit. Knowledge of the pathways of bile formation and secretion as well as the adaptive and protective mechanisms employed by hepatocytes and non-parenchymal cells could provide potential targets to develop small molecule therapies that might reverse or alleviate these conditions (Matrix Cell A2). Even minor improvements in disease severity may have enormous clinical benefits. Finally, several of the neonatal cholestatic syndromes and disorders of bilirubin metabolism are potentially fatal without liver transplantation. These conditions are appropriate targets for gene therapy. In particular, Crigler-Najjar syndrome, in which the liver is completely normal except for the inability to conjugate bilirubin, is a disorder for which gene therapy to reconstitute UDP-glucuronosyl transferase activity is an appropriate and potentially achievable research goal (Matrix Cell C3).

Steps to Achieve Research Goals

Many of the basic research goals in the area of bile, bilirubin, and cholestasis research are most appropriately approached through individual, investigator-initiated research projects. Elucidation of normal pathways of bile salt, lipid, cholesterol, organic solute and bilirubin uptake, synthesis, metabolism, transport, and secretion require extensive cell biological approaches and animal models. Identification of new animal models for cholestatic liver disease might be aided by the use of rapid screening methods for serum bilirubin and bile salt levels on mice exposed to mutagens. Other approaches to animal models include the generation of transgenic mice and use of non-mammalian model organisms. These approaches would be stimulated by investigator collaborations.

Clinical studies in cholestatic liver disease would be facilitated by multicenter networks of investigators with expertise in the areas of pediatric liver disease, liver disease of pregnancy, and drug-induced liver disease. The Biliary Atresia Research Consortium (BARC) could be supportive of this effort through the acquisition of large numbers of

clinically well-defined cases of neonatal liver disease. BARC could be expanded to encourage enrollment of these other severe forms of pediatric cholestatic liver disease seen throughout the country. Studies on drug-induced liver disease could be aided by the Drug-Induced Liver Injury Network (DILIN), which is acquiring well-characterized cases of liver injury due to medications both prospectively and retrospectively. Cholestatic liver disease would be an important, specific focus of this network, and would be enabled by collaborations with investigators capable of performing genetic and molecular analyses of pertinent genes. Finally, attempts to help translate findings from basic research on cholestasis and bilirubin metabolism into practical approaches to diagnosis, prevention and therapy of these diseases are of great importance. Funding of studies aimed at developing safe and effective means of gene therapy for cholestatic liver disease can be considered a high priority.

Matrix of Research Goals in Bile, Bilirubin and Cholestasis

	Short-term Goals (0-3 years)	Intermediate-term Goals (4-6 years)	Long-term Goals (7-10 years)
High Risk	**A3. More fully define the normal fetal development and maturation of bile salt and bilirubin metabolic pathways.**	**B3. Develop drug therapy that stimulates bilirubin metabolic pathways or interferes with bilirubin production in newborn.**	**C3. Develop effective gene therapy for at least one form of severe, neonatal cholestasis or hyperbilirubinemia.**
Intermediate Risk	**A2. Define structure-function relationships of genes involved in cholestatic liver diseases and identify potential targets for therapy.**	**B2. More fully elucidate the normal pathways of bile salt, lipid and organic solute uptake, synthesis, transport & secretion in hepatocytes. Define the pathways and regulation of hepatic cholesterol synthesis and secretion.**	**C2. Define molecular basis and means of screening for or diagnosing acquired or adult forms of cholestatic liver disease such as cholestasis of pregnancy, sepsis, or total parenteral nutrition.**
Low Risk	**A1. Identify molecular causes of all forms of PFIC.**	**B1. Define whether polymorphisms of major bile transporters are involved in drug-induced cholestatic liver disease.**	**C1. Define molecular basis of pruritus & identify targets for potential therapies.**